Published in final edited form as:
J Cardiovasc Transl Res. 2014 March ; 7(2): 203–211. doi:10.1007/s12265-013-9515-7.

Coagulation, Protease Activated Receptors and Viral Myocarditis

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Abstract

The coagulation protease cascade plays an essential role in hemostasis. In addition, a clot contributes to host defense by limiting the spread of pathogens. Coagulation proteases induce intracellular signaling by cleavage of cell surface receptors called protease-activated receptors (PARs). These receptors allow cells to sense changes in the extracellular environment, such as infection. Viruses activate the coagulation cascade by inducing tissue factor expression and by disrupting the endothelium. Virus infection of the heart can cause myocarditis, cardiac remodeling and heart failure. Recent studies using a mouse model have shown that tissue factor, thrombin and PAR-1 signaling all positively regulate the innate immune during viral myocarditis. In contrast, PAR-2 signaling was found to inhibit interferon-β expression and the innate immune response. These observations suggest that anticoagulants may impair the innate immune response to viral infection and that inhibition of PAR-2 may be a new target to reduce viral myocarditis..

Keywords

tissue factor; protease activated receptor; thrombin; myocarditis; coxsackievirus B3; virus infection; innate immune response

During infection the immune system is activated to eliminate the pathogen. The coagulation system functions as part of the immune response by forming a clot that limits dissemination of pathogens. Importantly, there is crosstalk between coagulation and inflammation. In this review, we discuss recent findings on the contribution of activation of coagulation and the clotting cascade and protease activated receptors to early responses to viral infections particularly viral myocarditis.

Myocarditis

Myocarditis is defined as inflammation of the heart muscle which can be caused by different insults, such as infections, autoimmune reactions, toxins and adverse drug reactions. Infections with enteroviruses, adenoviruses and other viruses can cause viral myocarditis. One of the first viruses identified to cause viral myocarditis was coxsackievirus B3 (CVB3) [1,2]. Viral myocarditis can be divided in three phases [3,4]. The early phase (day 0–4 post infection) is defined as early virus replication in various organs, such as pancreas, liver and heart. During this phase cardiac injury is caused by the virus itself. In the acute phase of myocarditis (days 7 to 10 post infection) there is infiltration of immune cells into the heart and increased cytokine/chemokine expression that controls viral replication. At this stage

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COMPETING INTERESTS STATEMENT: None

cardiac injury is mediated by immune cells, such as auto-reactive T cells. In the last phase (after day 15 post infection) the virus is eliminated and inflammation subsides. However, viral genomes can persist in the heart. Complications of viral myocarditis can occur in early and late stages of the disease and include pathologic remodeling that can lead to impaired heart function, dilated cardiomyopathy (DCM) and heart failure [5,6].

Innate immune response in myocarditis

The innate immune response is a first line defense system to infection by pathogens. Toll-like receptors (TLRs) are a family of innate immune receptors that play a central role in host defense by recognizing pathogen-associated molecular patterns (PAMPs) [7]. Viral infections are detected by different receptors, including the endosomal receptor TLR3. TLR3 is activated by dsRNA from dsRNA viruses and replication of ssRNA viruses. The importance of the different innate and adaptive immune responses in viral myocarditis has been reviewed by others [8–10]. Studies with mice found that increased virus-specific IgGs are detectable from day 7 onwards post CVB3 infection [11]. This indicates that innate immunity has a major role in antiviral defense [10].

Experimental myocarditis studies in mice infected with either CVB3 or CVB4 have shown a role of TLR3 and its adapter Toll/IL-1 receptor/resistance (TIR)-domain-containing adapter-inducing interferon-β (TRIF) [12–16]. Activation of TLR3 leads to the expression of interferons (IFNs), which play a crucial role in the early innate immune response to viruses [17]. IFNs are divided into type I (IFN-α and IFN-β), type II (IFN-γ) and type III (IFN-λ). Type I, but not type II, IFN signaling has been shown to be required for an innate immune response to CVB3 infection [18,19]. Importantly, administration of IFN-β reduces CVB3-induced myocarditis in mice and humans [16,20–22]. IFN-α and -β are expressed at low levels by numerous cell types but expression is increased in virally infected cells. IFNs stimulate the expression of IFN-stimulated genes (ISGs) that help eliminate the virus [23]. The ISG 2’-5’ oligoadenylate synthetase (OAS)/RNase L inhibits CVB3 replication by cleavage of viral ssRNA [18]. Other ISGs are chemokines, such as CXCL10, and these recruit natural killer (NK) cells to the site of infection [24]. Notably, CXCL10 deficiency in mice is associated with increased cardiac injury after infection [12,24]. In viral myocarditis, NK cells are one of the first cell types infiltrating the heart to limit viral replication and spread [25].

Blood Coagulation

Tissue factor (TF) is the primary initiator of coagulation protease cascade [26]. It is the cellular receptor and cofactor for factor VII/VIIa (FVII/VIIa). TF is expressed by cells within the vessel wall and is essential for hemostasis [27,28]. It is also expressed by activated monocytes and this response is thought to be part of the innate immune response to limit dissemination of pathogens [29,30]. In addition, the clotting system activates participates in host defense. For instance, fibrin(ogen) engagement of the integrin Mac1 on macrophages is required for efficient clearance of *Staphlococcus aureus* in mice [31]. Furthermore, fibrin(ogen) modulates natural killer cell activity [32].

Activation of coagulation in viral infections

Viral infections lead to the induction of TF expression in various cell types that are exposed to blood and this leads to activation of the coagulation cascade [4,33–37]. Interestingly, the dsRNA analog, polyinosinic-polycytidylic acid (poly IC), is a synthetic ligand for TLR3 and when administered to mice induces TF expression in endothelial cells and a hypercoagulative state [38]. Zare et al. observed that TF expression in murine splenocytes was increased by ssRNA (poly I) but not dsRNA (poly IC) [39]. This induction was

MyD88-dependent and IFN-independent [39]. Furthermore, type I and III IFN expression was associated with activation of coagulation [40]. However, IFN-α administration reduced platelet reactivity and ventricular thrombus formation during CVB3 myocarditis [21,41]. Furthermore, proinflammatory cytokines produced during viral infections may increase TF expression in endothelial cells and monocytes [4,35,42].

Activation of coagulation in myocarditis

Heart failure and DCM induced by viral myocarditis is associated with increased risk of thrombosis [43]. Clinical case reports have described an increased incidence of ventricular fibrin deposition and thrombi during acute myocarditis [44–49]. The presence of ventricular thrombi during myocardial inflammation may be due to TF expression by the endocardium [46,50]. We and others have observed thrombosis in the heart in mouse models of myocarditis [4,21,37,43,51,52]. One study reported increased TF expression in the endocardium of patients with myocardial inflammation that was associated with immune cells, most likely $CD45^+$ leukocytes (activated T cells) [53]. Consistent with this, we observed an association between TF expression and markers of activated endothelial cells in heart biopsies of patients with suspected myocarditis [4]. Furthermore, we showed that CVB3 infection induces myocardial TF expression [4,37]. Our data suggest an acute but no longer lasting inflammation of the heart leads to increased TF expression that triggers thrombosis [4,42,43,54]. In the chronic phase of heart failure, other parameters, such as platelet reactivity and blood stasis, may contribute to the enhanced risk for thrombotic events, as seen in patients with chronic DCM [4,44]. It has been suggested that anti-thrombotics should be used in patients with active cardiac inflammation caused by myocarditis [42,46,55–58].

Protease-activated receptors

There is cross-talk between the coagulation cascade and inflammatory response that is mediated, in part, by protease activated receptors (PARs) [59]. PARs belong to the family of G protein-coupled receptors [59]. There are four PARs (PAR-1 to -4). Cleavage of the N-terminal sequence of the receptor reveals a new N-terminal sequence that acts as tethered ligand by binding to the receptor and inducing its activation. A wide range of serine proteases, including immune cell-derived proteases and coagulation proteases can either activate or inactivate PARs which is dependent on cell type and post-translational modification of the receptor (Table 1). Thrombin is the primary activator of PAR-1, 3 and 4 whereas tryptase and trypsin are the primary activators of PAR-2 [60,61]. PARs are expressed by many cell types in the body and their expression patterns are comparable in different species. However, one important species specific difference in PAR expression exists; human platelets express PAR1 and PAR4 whereas mouse platelet express PAR3 and PAR4 [62]. The role of PARs in different diseases and immune responses has been reviewed by others [63,64]. PAR activation can be either pro-inflammatory or anti-inflammatory responses depending on the activator and cell type [63,64]. Moretti et al. proposed a dual-sensor system whereby an infection is detected by TLRs recognizing PAMPs and PARs being activated by extracellular proteases [65]. TLR-dependent signaling was affected by PAR expression/activation and *vice versa* [65].

Serine proteases in viral myocarditis

Besides coagulation proteases, other serine protease are expressed and released by stromal and circulating immune cells during infection [64]. Inhibitors of serine proteinases a routinely used to reduce activation of coagulation and inflammation in the clinic, such as during cardiac surgery. One of these inhibitors is the naturally occurring proteinase inhibitor

aprotinin. Administration of aprotinin reduced thrombin generation and PAR-1 dependent signaling [66]. In addition, trypsin is ectopically expressed by cardiac cells after ssRNA virus infection [67,68]. Aprotinin reduced trypsin-mediated signaling and viral replication in a model of influenza A myocarditis [67,68]. Trypsin stimulation of cells inhibited IFN responses after influenza A infection [69], which may be via PAR-2 activation. In addition, trypsin may increase the infectivity of influenza A via the cleavage of the hemagglutinin protein [70]. Aprotinin also reduced inflammation by preventing trypsin activation of PAR-4 and recruitment of neutrophils [71]. The known inhibitory action of aprotinin against trypsin as well as thrombin suggests that several pathways may be affected by aprotinin during virus infection.

Neutrophils secrete several proteases, such as cathepsin G, which activate PAR-1 and -4 [72]. Neutrophils are involved in clearing CVB3 from the heart [73]. Further, neutrophils contribute to T cell mediated pathologic responses in the heart during autoimmune myocarditis [74]. Cathepsin G and neutrophil elastase inactivate PAR-2 on human lung epithelial cells [75]. In addition, elastase can activate PAR-1 on epithelial cells [76]. The neutrophil elastase inhibitor ZD0892 was shown to reduce viral myocarditis by inhibiting immune cell infiltration without effecting the viral load [77]. Other proteases, such as matrix metalloproteinases (MMP), have been implicated in viral myocarditis progression [78]. MMP1 and MMP13 are able to activate PAR-1 [37,79,80]. Cardiac cells express MMP13 [79]. We found that a pan-MMP inhibitor or an MMP13 inhibitor reduced TLR3 dependent expression of CXCL10 by cardiac fibroblasts *in vitro* (Figure) [37]. In addition, line with these findings, MMP13 inhibition increased the virus levels and cardiac injury in CVB3 infected mice [37].

Mast cells provide another rich source of serine proteases, such as tryptase and chymase, which are activators of PAR-2 [81,82]. Mast cells degranulate within six hours of CVB3 infection [83]. Furthermore, mast cell deficient mice develop significantly less myocarditis than control littermates when infected with encephalomyocarditis virus [84]. However, the effect of inhibitors of mast cell tryptase, such as by protamine [85], and other commercially available tryptase inhibitors, such as APC366 [86], has not been studied.

Interestingly, the virus genome itself can encode proteases [87]. Enterovirus protease 3C was shown to exhibit papain/chymase-like activity and inhibition of enterovirus protease 3C reduced virus levels [88]. Papain can stimulate PAR-2 dependent signaling in T-cells [89]. Furthermore, papain-like viral proteases blocked dsRNA-induced IFN-β expression by interfering with IRF3 activation [90]. In addition, chymase pretreatment of fibroblasts rapidly inhibited the ability of these cells to respond to thrombin [91]. The data suggest that virally encoded papain/chymase-like proteases may activate PAR-2 and inactivate PAR-1 and thus reduce anti-viral innate immune responses.

PARs and viral myocarditis

PAR-1 is expressed by cardiomyocytes, cardiac fibroblasts, smooth muscle cells, endothelial cells and leukocytes [60]. During viral infection, thrombin mediated PAR-1 activation was linked to increased susceptibility to herpes viruses of endothelial cells in vitro [92]. We recently investigated the role of PAR-1 in a mouse CVB3 myocarditis model. Early after infection, we observed reduced expression of IFN-β and the IFN response gene CXCL10 as well as NK cell infiltration into the heart in PAR-1 deficient mice compared to wild-type mice [37]. These changes were associated with a significant increase in viral load in the heart and increased cardiac injury 8 days post infection [37]. Using bone-marrow transplantation, we found that PAR-1 on non-hematopoietic cells played a major role in the immune response [37]. *In vitro* studies revealed that PAR-1 stimulation on cardiac

fibroblasts enhanced TLR3-dependent p38 activation and expression of IFN-β and CXCL10 (Figure) [37]. Further, PAR-1 activation was associated with increased NK cell activity of murine splenocytes [37]. This is in agreement with an earlier study showing NK cell activity can be increased by thrombin [93].

PAR-2 is expressed by cardiomyocytes, cardiac fibroblasts, smooth muscle cells, endothelial cells and immune cells [60,64]. TF-dependent PAR-2 activation increased herpes simplex virus infection of endothelial cells *in vitro* [94]. In collaboration with Rauch's group we found that PAR-2 deficient mice were protected from CVB3 myocarditis compared to infected wild type mice [95]. An absence of PAR-2 was associated with reduced viral load in the heart and myocarditis. Interestingly, hearts and isolated cardiac fibroblasts of PAR-2 deficient mice expressed higher levels of IFN-β [95]. *In vitro* experiments revealed that PAR-2 activation reduced TLR3-dependent expression of IFN-β and IFN-responses genes in cardiac fibroblasts as well as epithelial cells [95,96]. This observation may be due to a direct interaction of PAR-2 with TLR3 which subsequently leads to inhibition of TLR3 signaling [95]. Others showed that PAR-2 contains a TIR domain that may interact with adaptor proteins, such as MyD88 and TRIF [97]. In contrast to the results with TLR3, PAR-2 enhances TLR4 dependent signaling [97,98]. With regard to myocarditis, TLR4 was shown to contribute to disease progression [99–102]. PAR-2 expression was positively correlated with myocardial inflammation and negatively with IFN-β expression and heart function in hearts of patients with non-ischemic cardiomyopathy [95]. Furthermore, Pan et al. showed that PAR-2 inhibition reduced influenza replication in cardiomyocytes *in vitro* [68].

PAR-4 is known as the main signaling receptor for thrombin on mouse platelets [62]. Platelets play a central role in hemostasis and thrombosis [103]. However, they also contribute to other processes, such as inflammation and immunity [104,105]. PAR-4 is also expressed by cardiomyocytes and smooth muscle cells in the heart as well as circulating immune cells [60,64,106]. It was proposed that PAR-4 mediates a pro-survival signal within the heart and that a lack of PAR-4 increases apoptosis and loss of cardiomyocytes [60]. The role of PAR-4 in infections has not been investigated in detail. However, we observed increased plasma cardiac troponin I levels in PAR-4 deficient mice compared to wild-type mice 8 days after CVB3 infection (Antoniak and Mackman, unpublished data). It is not clear whether these findings were due to activation of PAR-4 on platelets or other cells. Interestingly, non-steroid anti-inflammatory drugs (NSAIDs) including aspirin, decrease platelet reactivity. Mice studies and case reports suggested that the use of NSAIDs is associated with increased mortality in virus infections including myocarditis [107–110].

Anticoagulation

There are few studies reporting the effect of anticoagulation on viral myocarditis. Treatment with low molecular weight heparin begun either before or after virus inoculation reduced mortality after CVB3 infection of mice [57]. Heparin reduced collagen deposition and fibrosis in later stages of myocarditis but did not affect viral replication. However, heparin increased cardiac inflammation in the acute phase of infection [57]. The mechanism of this effect is not certain but may be due to reduced fibrin deposition and fibrosis [56]. Importantly, heparin also has anti-adhesive activity that may explain some of the effects. We observed increased virus levels and cardiac injury 8 days after CVB3 infection of mice treated with either a TF inhibitor or thrombin inhibitor compared to control treated mice [37]. These results suggested that TF mediated thrombin generation enhances antiviral responses that limit cardiac injury.

Schnitt et al. have suggested the use of a FXIII inhibitor or a fibrinolytic in viral myocarditis to reduce deposition of cross-linked fibrin and fibrin-mediated fibrosis [56]. However, our

results in a mouse model suggested that upstream coagulation proteases, such as thrombin, play a protective role in viral myocarditis [37]. One important question is does the use of anticoagulants increase the susceptibility and severity of viral myocarditis? Warfarin has been the main stay for long-term anticoagulation for many years. More recently, the thrombin inhibitor dabigatran etexilate and the factor Xa inhibitor rivaroxaban have been approved for long-term anticoagulation [111]. While the risk of thrombosis is reduced in patients our results with a mouse model suggest that reduced thrombin activity may increase the susceptibility to viral infection [37]. In response to our publication [37], investigators from the RE-LY study stated that "Pradaxa® (dabigatran etexilate) did not increase the incidence of virus infections when compared to warfarin" [112]. The authors further noted that other anticoagulants, such as warfarin or FXa inhibitors, should also increase the susceptibility of virus infections [112]. Rauch's group treated wild-type mice with the FXa inhibitor fondaparinux sodium and observed increased cardiac inflammation but reduced fibrosis and improved heart function 8 days after CVB3 infection compared to the placebo treated group [113]. At present, it is unclear if anticoagulation affects the frequency and intensity of viral infection.

Conclusions

Recent studies indicate that coagulation proteases and PARs modulate the innate immune responses to virus infections in mouse models, including myocarditis (Table 2). Interestingly, PAR-1 enhances while PAR-2 inhibits TLR3 signaling in cardiac fibroblasts. The activators of PAR-2 in myocarditis are not yet characterized. Importantly, our findings suggest that anticoagulation may increase the risk and severity of certain virus infections. Furthermore, inhibition of PAR-2 could be a new interventional strategy to treat viral myocarditis. One must be cautious in extrapolating from mice studies to humans since human myocarditis is highly diverse [114]. With regards to the effect of anticoagulation, there are no current clinical trials to determine if anticoagulants increase the incidence and severity of virus infections. One would need to compare infections in demographic, co-morbidity matched controls with or without anticoagulant therapy. Secondly, it is possible that divergent results will be observed with different anticoagulants. Further studies are needed to investigate the role of coagulation proteases, PARs and platelets in different virus infections.

Clinical Relevance

Basic research studies have increased our understanding of the molecular mechanism of viral myocarditis and revealed promising pathways for interventions. Our data suggest a potential new treatment option for viral myocarditis is inhibition of PAR-2 [95]. Several studies has described the development of PAR-2 inhibitors that are potential candidates for clinical trials [115,116]. However, to date the new knowledge on viral myocarditis has not resulted in any substantial improvement in the treatment of viral myocarditis [114]. This is likely due to the fact that viral myocarditis is often diagnosed too late after infection. One positive note is that Kuhl and coworkers showed that IFN-β treatment reduced virus levels and improved heart function and survival in patients with enterovirus myocarditis [20,22].

Acknowledgments

We thank Dr. Julie C. Williams for her careful and critical review of this manuscript. This work was supported by grants from the Myocarditis Foundation (to S. Antoniak) and the National Institutes of Health (to N. Mackman, HL119523).

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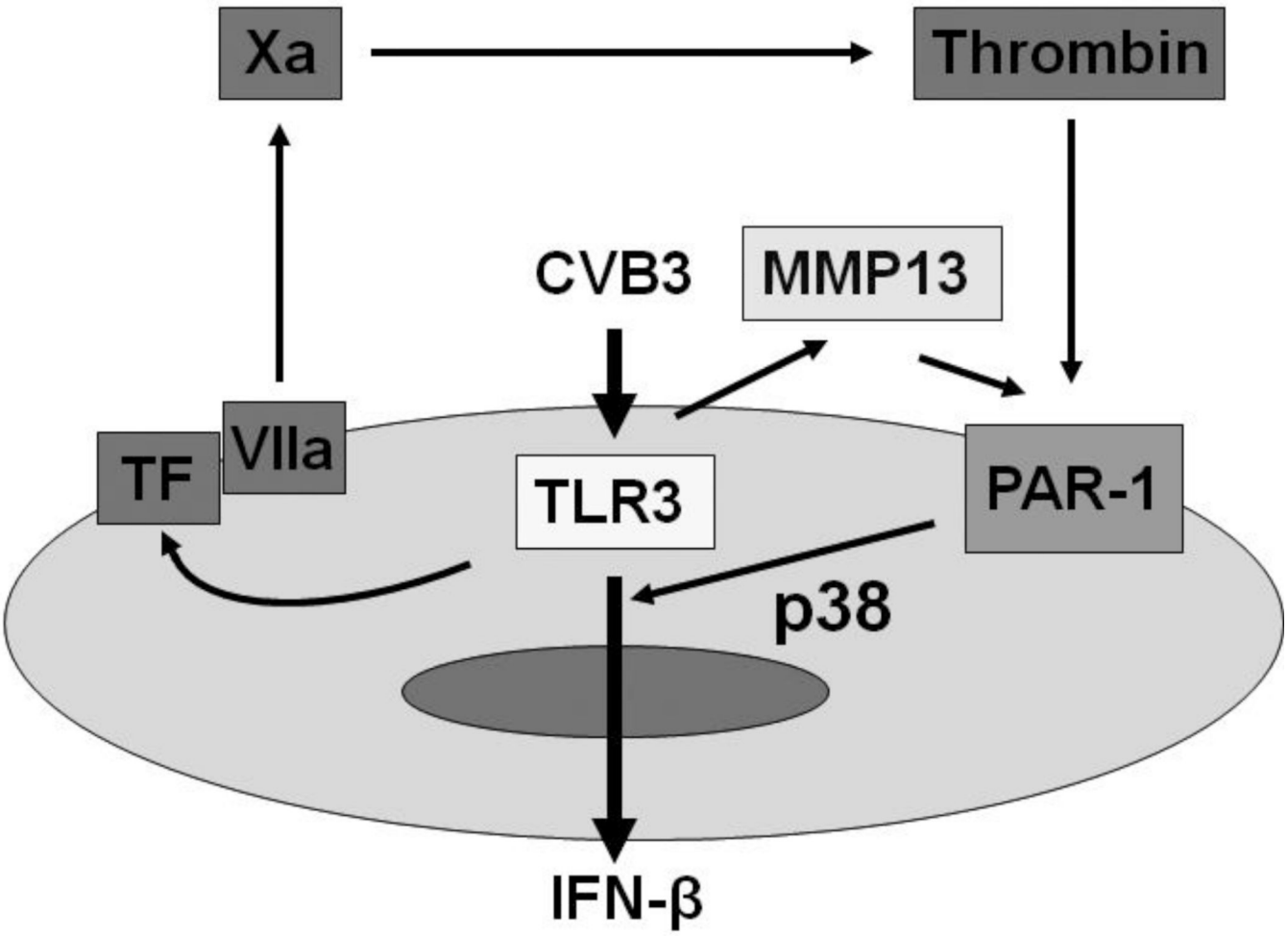

Figure. The thrombin PAR-1 pathway enhances TLR3-depdendent innate immune responses in CVB3 myocarditis

CVB3 infection leads to increased TF expression and activation of coagulation. Thrombin mediated PAR-1 cleavage contribute to TLR3 dependent IFN-β due to increase in p38 activation on cardiac cells, such as cardiac fibroblasts. Additionally, PAR-1 can be activated by MMP13 and enhances TLR3 signaling during viral infection.

Table 1

Activating and inactivating proteases of PAR-1 and PAR-2

Protease	Effect on PAR	Reference
Thrombin	Primary activator of PAR-1	[59]
FXa	Activates PAR-1 and PAR-2	[60,61]
FVIIa	Activates PAR-2	[60,61]
MMP1 and MMP13	Activates PAR-1	[37,79,80]
Tryptase	Primary activator of PAR-2	[82]
Trypsin	Activates PAR-1 and PAR-2	[61]
Cathepsin G	Activates PAR-1	[72]
	PAR-2 inactivation on lung epithelial cells	[75]
Chymase	Reduces responses of fibroblasts to thrombin possible due to PAR-1 deactivation	[91]
	Activates PAR-2	[81]
Elastase	Activates PAR-1 on lung epithelial cells	[76]
	PAR-2 inactivation on lung epithelial cells	[75]
Papain	Induces PAR-2 dependent signaling in T-cells	[89]

Table 2

Roles of PAR-1, PAR-2 and different proteases in viral myocarditis

Manipulation	Observation	Reference
PAR-1 deficient mice	Increased CVB3 myocarditis due to reduced INF-β responses	[37]
Overexpression of PAR-1 on cardiomyocytes *in vivo*	Reduced CVB3 myocarditis	[37]
PAR-2 deficient mice	Reduced CVB3 myocarditis	[95]
Thrombin inhibition with dabigatran etexilate in wild-type mice	Increased CVB3 myocarditis	[37]
MMP13 inhibition WAY170523) in wild-type mice	Increased CVB3 myocarditis	[37]
Fondaparinux (FXa inhibitor) in wild-type mice	Increased cardiac inflammation but improved heart function after CVB3 infection	[113]
Mast cell deficiency (loss of tryptase and chymase)	Reduced encephalomyocarditis virus myocarditis	[84]
Trypsin inhibition by aprotinin in wild-type mice	Reduced Influenza A virus myocarditis *in vivo* and *in vitro*	[67,68]
Neutrophil elastase inhibition by ZD0892 in wild-type mice	Reduced viral myocarditis by inhibiting immune cell infiltration	[77]
PAR-1 stimulation with TFLLR-NH(2)	Increased TLR3 dependent IFN-β and CXCL10 expression in cardiac fibroblasts *in vitro*	[37]
PAR-1 inhibitor (SCH79797), MMP13 inhibitor WAY170523) and pan-MMP inhibitor (GM6001)	Reduced TLR3 dependent production of CXCL10 in cardiac fibroblasts *in vitro*	[37]
PAR-2 activation and PAR-2 overexpression	Reduced TLR3 dependent IFN-β responses in cardiac fibroblasts and epithelial cells	[95,96]
PAR-2 inhibition by FSY-NH(2) or trypsin inhibition by aprotinin	Reduced influenza A virus replication in cardiomyocytes	[68]
Thrombin stimulation and PAR-1 expression	Increased susceptibility of endothelial cells to HSV	[92]
PAR-2 activation	Increased susceptibility of endothelial cells to HSV	[94]